# Appendix S
## DETECTION OF *ACTINOMYCETES* IN WATER

**Overview:**
The following is a method for isolation of Actinomycetes from water. The plating method used is the double agar layer (DAL) method, adopted from the American Public Health Association (2005).

**Media and reagents:**
***Cycloheximide***

- Antifungal antibiotic solution
    - 0.1 g cycloheximide (Sigma, St. Louis, MO, C7698-1G)) into 100 mL reagent water
    - Autoclave for 15 minutes
    - *Store at 4°C for up to six months*

***ISP Medium 1 (ISP#1) broth***

- Used for positive control broth culture.
- Make according to the directions on the bottle.
    - 8 g ISP#1(VWR, Pittsburgh, PA, DF0769-17) into 1 L reagent water
    - Heat to dissolve
    - Autoclave for 15 minutes
    - *Store at 4°C for up to 6 months*

***ISP Medium 2 (ISP#2) agar***

- Used for maintaining a refrigerator slant of *S. albus*
- Make according to the directions on the bottle
    - 38 g ISP#2 (VWR, Pittsburgh, PA, DF0770-17) into 1 L reagent water
    - Heat to dissolve
    - Autoclave for 15 min
    - Agar can be stored in dilution bottles for up to 6 months in the refrigerator

***Actinomycete Isolation Agar (AIA)***

- Used for making top agar and bottom agar plates for the samples and controls
- Make according to the directions on the bottle
    - 22 g AIA (VWR, Pittsburgh, PA, DF0957-17) into 1 L reagent water
    - Heat to boiling to dissolve completely
    - Add 5 g Glycerol and mix well
    - Dispense 100 mL into dilution bottles
    - Autoclave for 15 minutes
    - *Agar can be stored in dilution bottles for up to 6 months in the refrigerator.*

**Quality control:**
***Positive control***

- A positive control is plated every time samples are analyzed.
    - Prepare a positive control broth culture 1 week prior to sample analyis.

- Transfer a 1 µL loop full of *S. albus* from the refrigerator slant into a test tube containing 10 mL of ISP medium 1
- Incubate for 7 days at 28°C

- At time of sample analysis, create a $10^{-2}$ dilution of the positive control broth culture.
  - Plate the dilution following the directions for the DAL method. Add 2 mL of the $10^{-2}$ dilution in place of the sample.

***Negative control***

- A negative control is plated every time samples are analyzed to ensure the media is not contaminated.
- Follow the directions for the DAL method, except add 2 mL of sterile water instead of sample.

***Maintaining organism***

- *S. albus* is stored at 4°C on an ISP#2 slant.
- Transfer the organism to a new ISP#2 slant and every two months.

**Sample analysis:**

- Samples are typically analyzed directly and in a $10^{-1}$ dilution. If it is believed the actinomycete concentration is elevated, higher dilutions should be analyzed as well. A duplicate is also analyzed for each dilution.

*Double agar layer method*

1. For the water sample to be tested, make dilutions as followed:
   - Undiluted: Aliquot from sample bottle
   - $10^{-1}$: 11 mL of undiluted sample into 99 mL MI buffer
2. Pour AIA bottom agar into 100 mm plates
   - Approximately 20 mL per plate
   - Warm plates in 36°C incubator prior to analyzing sample
3. Prepare top agar
   - This is done in a 48°C waterbath
   - In sterile test tubes (one for each dilution, duplicates, and positive and negative controls) aliquot 17 mL of AIA
   - Add 1 mL of cyclohexamide to each test tube
4. Analyze sample
   - Add 2 mL of sample dilution (or positive or negative control)
   - Gently mix
   - Pipet 5 mL over a bottom agar plate. In order to spread mixture evenly before the agar hardens, swirl the plate while dispensing the mixture.
   - Repeat the analysis for each sample, dilution, and controls
5. Incubate at 28°C for 7 days.

**Counting colonies:**

- Ideal count is between 10−30 colonies. Plates with target colonies above this range often contain considerable non-target growth and are difficult to read.
- Identify actinomycetes by gross colony appearance. If necessary, verify by microscopic examination at a magnification of 50 to 100x.

- Characteristics to look for in an actinomycetes colony when compared to a nontarget typical colony:
    - Actinomycetes: An earthy, soil smell, opaque, sometimes chalky appearance in mature colonies, darker in the center and lighter farther from the center, irregular, fuzzy edge, of hyphal appearance, strong adherence to medium, and strong and leathery texture.
    - Typical nontarget colony: Shiny appearance, regular, smooth edge, uniform look throughout colony, weak adherence to medium, and soft texture.

**Documentation**

- Results of QC samples are recorded in the sample lab book.
- Record sample results on the Actinomycete bench sheet and store in the sample log book with the sample service request form.
- Records of media sterility are stored in the media log book.
- *S. albus* strain maintenance is recorded in LIMS

**Reference**

American Public Health Association, American Water Works Association, and Water Environment Federation, 2005, Standard methods for the examination of water and wastewater (21st ed.): American Public Health Association, part 9250, p. 9-109 to 9-111.

Sample No.: _____

# Laboratory analysis - Actinomycetes on AIA

Station Name: _____

Site Number: __ __ __ __ __ __ __ __ __ __ __ __ __ __ __

Date (mm/dd/yyyy): __ __ / __ __ / __ __ __ __ Time (military): __ __ __ __

*To be filled out by laboratory analyst:*

Analyzed by (initials): _____ Date analyzed: _____

Read by (initials): _____ Date out of incubator: _____

Date read: _____

| Sample size | Colony count A | Colony count B |
|---|---|---|
| Undilute (0.5 mL) | | |
| 1:2 (0.25 mL) | | |
| 1:10 (0.05 mL) | | |
| 1:100 (0.005 mL) | | |
| Negative control | | -- |
| Positive control (*Streptomycetes albus*) | | -- |

QA ID: _____

**Average result: _____ colonies/mL**

**Comments:**